AD_____

Award Number: DAMD17-02-1-0538

TITLE: Breast Cancer Metastasis to Bone Affects Osteoblast Differentiation

PRINCIPAL INVESTIGATOR: Robyn R. Mercer

CONTRACTING ORGANIZATION: The Pennsylvania State University
University Park, PA 16802-7000

REPORT DATE: May 2004

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	May 2004	Annual Summary (15 Apr 2003 - 14 Apr 2004)

4. TITLE AND SUBTITLE

Breast Cancer Metastasis to Bone Affects Osteoblast Differentiation

5. FUNDING NUMBERS

DAMD17-02-1-0538

6. AUTHOR(S)

Robyn R. Mercer

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

The Pennsylvania State University
University Park, PA 16802-7000

E-Mail: rrm150@psu.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

Breast cancer fatally metastasizes to bone and activates osteoclasts, cells that resorb bone, resulting in the formation of osteolytic lesions. Certain drugs, bisphosphonates, slow the action of osteoclasts, however, the bone lesions are not repaired.

The osteoblasts should be able to repair the lesions by synthesizing new bone matrix. Instead, these cells appear to be inactivated by breast cancer, and the lesions do not heal. The purpose of this proposal is to understand what happens to osteoblasts in the presence of breast cancer. We hypothesize that breast cancer cells prevent pre-osteoblasts from completely maturing to osteoblasts. Our goals are to examine the effects of breast cancer cells on osteoblast proliferation, differentiation, and mature function. Using an osteoblast cell line and metastatic breast cancer cells, we found that conditioned medium from breast cancer cells inhibited osteoblast differentiation, as demonstrated by an inhibition of alkaline phosphatase, bone sialoprotein, and osteocalcin mRNA expression, and an inhibition of mineralization. We found that these effects on differentiation are mediated through TGFβ present in the conditioned medium. We also found that MDA-MB-231 conditioned medium altered osteoblast adhesion. Over the next year, we will further characterize these observations.

14. SUBJECT TERMS

Bone, Metastasis, Breast cancer, Osteoblast Differentiation, Osetolytic Metastasis

15. NUMBER OF PAGES

9

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

INTRODUCTION

Breast cancer frequently metastasizes to bone where it disrupts the delicate balance between osteoblasts (the bone forming cells) and osteoclasts (bone resorbing cells). Osteolytic lesions form at the site of invasion as a result of osteoclast activation. Certain drugs, bisphosphonates, are presently being used in an effort to block osteoclast function. These drugs slow lesion progression; however, the surrounding osteoblasts appear to be inactive and do not repair the lesions. We hypothesize that breast cancer cells alter the ability of osteoblasts to differentiate into mature, functional, matrix-producing cells. The aims of this proposal are 1) To determine if the growth phase of osteoblasts will be altered in the presence of breast cancer cells; 2) To determine if production of the osteoblast differentiation proteins alkaline phosphatase, bone sialoprotein and osteopontin are altered during the differentiation of osteoblasts in the presence of breast cancer cells or conditioned medium; 3) To determine if osteoblasts retain the ability to lay down a bone matrix and produce mature osteoblast proteins after exposure to breast cancer. To test this idea, conditioned medium from a bone-metastatic breast cancer cell line, MDA-MB-231, was cultured with osteoblasts that can differentiate in culture. These osteoblasts have been analyzed for their ability to proliferate, secrete matrix proteins, and form a mineralized matrix in the presence of MDA-MB-231 conditioned medium.

BODY

The initial cell line to be used in this proposal was a human Fetal Osteoblast line called hFOB 1.19. The literature states that this is an immature osteoblast cell line that can be induced to differentiate in culture. However, as was explained in the previous progress report (May, 2003), we experienced difficulty in getting this line to differentiate. After spending an exhaustive two years toward this effort, we chose to pursue a different cell line, MC3T3-E1. This is an immature, murine osteoblast cell line that has also been reported to differentiate in culture. At the time of the last progress report, studies with this new cell line had just begun. Since that time, we have found it to be an excellent cell line for studying differentiation, as will be described in this report.

Task 1: To determine if the growth phase of osteoblasts will be altered in the presence of breast cancer cells. (Months 1-6)

a. ***Breast cancer cell conditioned media will be added to osteoblasts at the beginning, middle and end of the hFOB 1.19 growth cycle. Osteoblasts will be isolated, stained with crystal violet, and cell number determined. Growth will be measured at regular intervals on days 1,3,5 and 7. (month 1)***

MC3T3-E1 osteoblasts were cultured with conditioned medium from a human bone metastatic cell line, MDA-MB-231. 50% conditioned medium or 50% vehicle control medium (serum-free medium) was added to MC3T3-E1s 24 hours after plating. MC3T3-E1 cell number was determined indirectly by analyzing mitochondrial activity with the Promega Cell Titer assay. Using this procedure, we found that MDA-MB-231 conditioned media caused a modest increase in MC3T3-E1 cell number (Figure 1). As an additional control, conditioned medium from a fibroblast cell line, NIH 3T3, was also cultured with the MC3T3-E1 cells. Cells cultured with either vehicle control medium (VM) or 3T3 fibroblast conditioned (3T3 CM) medium had similar growth rates.

b. ***Task 1b-1c:*** These aims have not yet been pursued.

c. ***Determine if breast cancer cells or conditioned medium inhibits the growth of primary osteoblasts. (months 4-6)***

It is difficult for our lab to obtain human primary osteoblasts. On two separate occasions, we have received a flask of cells from a collaborator. MDA-MB-231 conditioned medium also caused a modest, 2-fold increase in cell number, similar to that observed with MC3T3-E1 osteoblasts.

Task 2: To determine if the production of alkaline phosphatase, bone sialoprotein, and osteopontin are altered during the differentiation of osteoblasts in the presence of breast cancer cells or conditioned medium.
(Months 7-28)

a. ***Detect alterations in osteoblast mRNA production of the differentiation proteins alkaline phosphatase, bone sialoprotein, and osteopontin caused by the addition of breast cancer cell conditioned media. Conditioned media will be added during the beginning, middle, and late stages of osteoblast differentiation, and analysis will be performed using Northern blot and RT-PCR. (months 7-9)***

We analyzed MC3T3-E1 expression of a selected group of osteoblast proteins, including bone sialoprotein, osteocalcin, osteonectin, and alkaline phosphatase activity in the presence of MDA-MB-231 conditioned medium. In preliminary studies, we established the pattern of expression of these proteins in the MC3T3-E1 cells in our laboratory. Using RT-PCR, we found that bone sialoprotein expression was first observed between 7 and 9 days of culture, while osteocalcin expression was not detected until about 13 days. Osteonectin mRNA was detected in immature osteoblasts (3 days) and levels began to increase after 15 days (data not shown).

Therefore, MC3T3-E1 osteoblasts were cultured with MDA-MB-231 conditioned medium and assayed for mRNA levels of these proteins. mRNA was isolated every other day for up to 30 days of culture. Expression levels for both bone sialoprotein and osteocalcin were greatly downregulated in response to MDA-MB-231 conditioned medium (Figure 2, shown after 15 and 23 days of culture). Expression levels of mRNA for osteonectin were initially equivalent to cells cultured in vehicle control medium. However, after 15 days of culture, mRNA levels in control cells were enhanced, but this did not occur in cells cultured with the cancer conditioned medium. Instead, osteonectin mRNA expression remained near the basal level in these cells, even after 30 days. Although these results were obtained using 50% conditioned medium, the same effects were seen in a dose dependant manner with 5% to 35% conditioned medium (data not shown).

We also examined alkaline phosphatase activity. The cells were cultured with 50% conditioned medium from either MDA-MB-231 cells or NIH 3T3 fibroblasts, or with 50% vehicle control medium for 35 days. NIH 3T3 fibroblast conditioned media were used as a negative control. To assay for alkaline phosphatase activity, the cells were fixed in formaldehyde and stained with Napthol AS-BI Phosphate and Fast Blue RR Salt. This solution provides a substrate for alkaline phosphatase that when cleaved, turns blue. MC3T3-E1 cells had no detectable alkaline phosphatase activity when cultured with MDA-MB-231 cell conditioned media, but activity was not affected by NIH 3T3

fibroblast conditioned media or vehicle control media (Figure 3A and B). Cells were examined at various times up to 35 days of culture.

Tasks 2b-2c: These experiments have not yet been addressed.

Tasks 2d-2e: Initially, we wanted to determine if breast cancer conditioned medium could alter either the protein expression or mRNA levels of these osteoblast differentiation proteins. Because we found a complete inhibition in mRNA levels, we have not pursued looking at the protein expression.

Tasks 2f-2g: These experiments have not yet been addressed.

Task 2h: Using human primary osteoblasts, alkaline phosphatase activity was assayed as described for MC3T3-E1 osteoblasts. We observed a similar trend in that there was an inhibition of alkaline phosphatase activity in osteoblasts treated with MDA-MB-231 conditioned medium (data not shown).

In addition to the tasks outlined above, we wanted to determine what factor present in the MDA-MB-231 conditioned medium could be responsible for the observed inhibition of differentiation. In referring to the literature, we found that transforming growth factor β (TGFβ) may be a good candidate. Therefore, MC3T3-E1 osteoblasts were cultured with MDA-MB-231 conditioned medium or vehicle control medium that was pre-incubated with a neutralizing antibody against TGFβ. In the presence of MDA-MB-231 conditioned medium and neutralizing TGFβ antibody, gene expression of bone sialoprotein, osteocalcin and alkaline phosphatase were all restored to control levels (data not shown).

Task 3: To determine if osteoblasts retain the ability to lay down bone matrix and produce mature osteoblast proteins (Type I collagenase and osteocalcin) after exposure to breast cancer. (months 29-32)

a. ***Add breast cancer cells or conditioned media to hFOBs as in task 2a and assay for bone nodule formation using Von Kossa staining of osteoblasts in co-culture, conditioned media, or a transwell system. (month 29)***

MC3T3-E1 cells mineralize in culture, which can be visualized using the Von Kossa stain. When cultured with conditioned medium from MDA-MB-231 cells, mineralization is greatly decreased compared to controls (vehicle control medium and NIH 3T3 fibroblast conditioned medium) (Figure 3C and D).

Task 3c: MDA-MB-231 conditioned medium inhibits osteocalcin expression, as described in Task 2a.

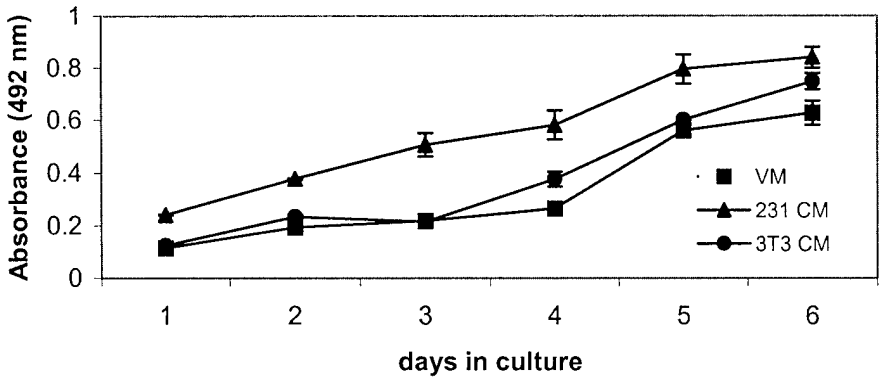
Task 4: Data analysis and thesis preparation. Task 4 is in progress.

In addition to the tasks outlined above, we have made further observations with the MC3T3-E1 cell line. When initially culturing these cells to determine MDA-MB-231 conditioned medium effects on mineralization, we noticed that the conditioned medium induced a change in cell morphology. Once MC3T3-E1 cells reach confluence, they lose their fibroblast morphology and begin to take on a patterned, cobblestone appearance. However, in MDA-MB-231 conditioned medium, the cells become very long and spindle shaped. Based on these changes in morphology, we looked at the actin filaments in the cells. MC3T3-E1 cells were cultured with MDA-MB-231 conditioned medium for 3 days, fixed with paraformaldehyde, and stained with phalloidin to identify f-actin. Stress fiber formation was evident in the control cells, but primarily punctate and cortical

staining was observed in the cells treated with conditioned medium (Figure 4). To further support alterations in stress fibers, the cells were examined for the presence of focal adhesion plaques using interference reflection microscopy. MC3T3-E1 cells cultured with MDA-MB-231 conditioned medium displayed a reduction in focal adhesion plaques (data not shown). Furthermore, we've found that the changes in stress fibers and focal adhesion plaques are mediated through PI3 kinase.

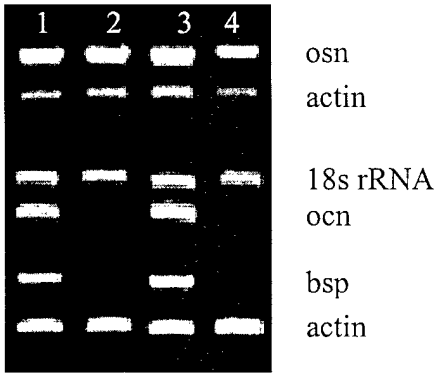
Progress in Program: From the time this proposal was submitted, all required course work and examinations have been completed. In June 2003, I was selected to attend the "Pathobiology of Cancer Workshop" sponsored by the American Association for Cancer Research.

Figure 1: Breast Cancer Conditioned Media Enhances MC3T3-E1 Cell Number



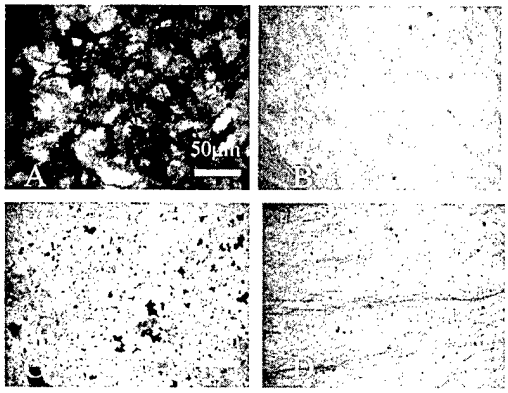
MDA-MB-231 or NIH 3T3 conditioned medium, or vehicle control medium (VM) were added to MC3T3-E1 osteoblasts 24 hours after plating. Cell number was determined using a cell viability assay. Results are representative of triplicate samples.

Figure 2: MDA-MB-231 Conditioned medium inhibits expression of bone sialoprotein (BSP), osteocalcin (OCN), and osteonectin (OSN)



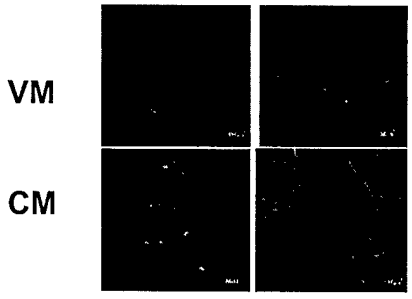
MC3T3-E1 osteoblasts were cultured with 50% vehicle control medium or 50% MDA-MB-231 conditioned medium for up to 30 days. RNA was isolated and analyzed using RT-PCR for bone sialoprotein (bsp), osteocalcin (ocn), and osteonectin (osn). Lane 1: vehicle control medium day 15; lane 2: MDA-MB-231 conditioned medium day 15; lane 3: vehicle control medium day 23; lane 4: MDA-MB-231 conditioned medium day 23.

Figure 3: MDA-MB-231 Conditioned Medium inhibits Alkaline Phosphatase Activity



MC3T3-E1 osteoblasts were cultured with 50% MDA-MB-231 conditioned medium for 35 days. Cells were fixed and assayed for alkaline phosphatase activity or for mineralization using Von Kossa. A) Alkaline phosphatase activity of cells cultured with vehicle control medium; B) Alkaline phosphatase activity of cells cultured with MDA-MB-231 conditioned medium; C) Von Kossa staining of cells cultured with vehicle control medium; D) Von Kossa staining of cells cultured with MDA-MB-231 conditioned medium.

Figure 4: MDA-MB-231 Conditioned Medium prevents stress fiber formation in MC3T3-E1 cells



MC3T3-E1 cells were cultured with conditioned medium from MDA-MB-231 cells and stained with phalloidin. Note the punctate staining in cells treated with MDA-MB-231 conditioned medium (CM) versus control cells.

Key Research Accomplishments:

- Discovered that breast cancer conditioned medium inhibits osteoblast differentiation
- This inhibition in differentiation is mediated through TGFβ
- Breast cancer conditioned medium disrupts cell adhesion, as seen through altered stress fibers and reduction in focal adhesion plaques

Reportable Outcomes: (abstracts)

Mercer, R.R., Gay, C.V., Welch, D., Mastro, A.M. (2003) American Association for Cancer Research Annual Meeting "Identification of mechanisms involved in breast cancer induced apoptosis of osteoblasts" Proceedings of the 94th Annual Meeting of the American Association for Cancer Research, vol. 44:2412.

Mercer, R.R., Gay, C.V., Welch, D., Mastro, A.M. "Breast cancer cells downregulate alkaline phosphatase production in osteoblasts" Oncology (2003) 17, suppl 3 pg 54.

Mercer, R.R., Welch, D., Gay, C.V., Mastro, A.M. (2003) IVth International Conference on Cancer-Induced Bone Diseases "Breast cancer skeletal metastases affect osteoblast function.

Mercer, R.R., Chislock, E.M., Miyasaka, C., Welch, D., Gay, C.V., Mastro, A.M. (2004) American Association for Cancer Research Annual Meeting "Breast cancer skeletal metastases affect osteoblast function" vol. 45:5190.

Mercer, R.R., Welch, D., Gay, C.V., Mastro, A.M. AACR Pathobiology of Cancer workshop: *The Edward A. Smuckler Memorial Workshop* "Breast cancer skeletal metastases induces osteoblast apoptosis"

Publications:

Harms, J.F., Welch, D., Samant, R.S., Shevde, L.R., Miele, M.E., Babu, G.R., Melly, R., Beck, L.N., Kent, J., Gilman, V.R., Sosnowski, D.M., Campo, D.A., Gay, C.V., Budgeon, L.R., Christensen, N.D., **Mercer, R.R.**, Jewell, J., Mastro, A.M., Donahue, H.J., Erin, N., Paquette-Straub, C., Griggs, D.W., Kotyk, J.J., Pagel, M.D., Westlin, W.F., Rader, R.K. "A small molecule antagonist of the $\alpha_v\beta_3$ integrin suppresses MDA-MB-435 skeletal metastasis." Clinical and Experimental Metastasis (2004) in press.

Mastro, A.M., Gay, C.V., Welch, D., Donahue, H.J., Jewell, J., **Mercer, R.R.,** DiGirolamo, D., Chislock, E.M., Guttridge, K. (2004) "Breast Cancer Cells Induce Osteoblast Apoptosis: A Possible Contributor to Bone Degradation". J. Cell. Biochem. 91(2): 265-276.

Conclusions: Understanding how breast cancer cells affect osteoblasts following skeletal metastasis will be instrumental in finding new drug targets to not only treat osteolytic lesions, but to also prevent lesion formation. Thus far, we have learned that breast cancer cells alter the proliferation rate of immature osteoblasts and inhibit their differentiation pattern. The inhibition of differentiation is mediated through TGFβ. In addition, MDA-MB-231 conditioned medium alters osteoblast adhesion, as demonstrated through altered stress fibers and a reduction in focal adhesion plaques. These changes are mediated through PI3 kinase, although the exact mechanism remains to be determined.

References: None

Appendices: None